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Chemical Pathology of Homocysteine I. Atherogenesis*

KILMER S. McCULLY, M.D.

Department of Veterans Affairs Medical Center, Providence, RI 02908

ABSTRACT

The atherogenic properties of homocysteine were discovered by observation of arteriosclerosis in children with homocystinuria caused by inherited deficiency of three different enzymes. Hyperhomocysteinemia is generally recognized as an independent risk factor for coronary, cerebral, and peripheral atherosclerosis. Hyperhomocysteinemia is caused by heterozygosity for homocystinuria, micronutrient deficiency from dietary imbalance, toxins, drugs, hormones, and other factors, explaining many key observations concerning the epidemiology of atherosclerosis. The etiological factors for atherosclerosis are believed to increase conversion of methionine to homocysteine thiolactone, the reactive cyclic internal lactone of homocysteine. The free amino groups of low density lipoprotein (LDL) are thiolated by homocysteine thiolactone, causing aggregation and increased uptake of LDL by macrophages, explaining lipid deposition in atheromas. Homocysteine thiolactone, released from homocysteinylated LDL within vascular wall, promotes intimal injury, oxidation of cholesterol and unsaturated lipids, platelet aggregation, thrombogenic factors, myointimal hyperplasia, deposition of sulfated glycosaminoglycans, fibrosis and calcification of atherosclerotic plaques.

Introduction

The degenerative diseases associated with aging, particularly arteriosclerosis and cancer, present a challenge to understanding of the correlations between fundamental aspects of their pathogenesis. The key to this understanding may have arisen from an obscure line of investigation earlier in the twentieth century that was concerned with the nutritional control of growth by several newly discovered amino acids containing sulfur. The purpose of this review to elucidate, within the limitations of present knowledge, the important role of the metabolism of these sulfur amino acids in atherogenesis, carcinogenesis, cellular and tissue function, degenerative diseases and aging.

This review of the chemical pathology of homocysteine is divided into three sections for publication in successive issues of *Annals of Clinical and Laboratory Science*. Section I, *Atherogenesis*, describes the discovery of methionine,

* Address reprint requests to: Kilmer S. McCully, M.D., Pathology and Laboratory Medicine Service, VA Medical Center, Providence RI 02908-4799.

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homocysteine, and homocysteine thiolactone, the discovery of the homocysteine theory of arteriosclerosis, and current knowledge concerning the role of homocysteine in the pathophysiology of atherogenesis. Section II, *Carcinogenesis and Homocysteine Thiolactone Metabolism*, describes altered methionine and homocysteine metabolism in carcinogenesis, the synthesis of antineoplastic homocysteine thiolactone compounds, the effect of homocysteine thiolactone on normal tissues, the hypothetical function of thioretinaco and thioretinamide in oxygen metabolism, adenosine triphosphate synthesis and phosphoadenosine phosphosulfate synthesis, and the importance of homocysteine thiolactone metabolism in the growth of animals. Section III, *Cellular Function and Aging*, describes current knowledge of the role of homocysteine metabolism in cellular respiration, cell division, growth and function of epithelia and connective tissues, lipid transport and metabolism, adenosyl methionine synthesis, the aging process, and degenerative diseases associated with aging.

While many of the observations and interpretations described in this review are directly supported by the evidence cited in the references, the reader is cautioned that an attempt has also been made to explain many of the alterations of homocysteine metabolism which occur in the degenerative diseases of aging. This attempt has necessitated the postulation of a number of hypotheses, speculations, and conjectures regarding the schemes presented in Section II, Figures 4 to 11, and in Section III, Figures 2, 4, and 5. In many respects, extensive further experimental investigation is needed to establish the validity of these schemes with certainty. In defense of this approach, many of these proposals are described in sufficient biochemical, metabolic, and pathophysiological detail, so that experimental methods for proving or modifying these proposals can be developed by investigators with an interest in this challenging area of clinical and laboratory science.

Discovery of Methionine, Homocysteine and Homocysteine Thiolactone

Methionine, one of the eight essential amino acids of mammalian nutrition, was discovered in broths of bacterial cultures by Mueller in 1922.[60] The essential nature of methionine in nutrition, its role in methyl group transfer, and its conversion to cystathionine, cysteine, taurine, and sulfate were discovered by DuVigneaud and coworkers in the succeeding two decades.[17] These studies clearly demonstrated that, while methionine is essential for supporting the growth of animals, the requirement for cysteine in supporting growth can be supplied by methionine, through the intermediate formation of cystationine.

In the course of their work, a new sulfur amino acid, homocysteine, was discovered and shown to support the growth of animals, provided that nutrients containing methyl groups, such as choline or betaine, were supplied.[5] At about the same time, the anhydride of homocysteine, the cyclic internal lactone, homocysteine thiolactone, was synthesized as the hydriodide salt by reaction of hydriodic acid with methionine.[2]

Chemical Properties of Homocysteine Thiolactone

Because of the reactive nature of its five membered lactone ring, homocysteine thiolactone possesses several important chemical properties that are not shared by cysteine. Since it has one fewer methylene carbon atom than homocysteine, cysteine cannot form a sta-

ble internal cyclic lactone. Homocysteine thiolactone results from the action of strong acid on homocysteine, forming a stable, ionized, water soluble salt.[73] Homocysteine thiolactone is resistant to oxidation by perchloric acid, forming a stable perchlorate salt that is soluble in chloroform-methanol or ethanol.[83] The ring structure of the perchlorate salt of homocysteine thiolactone is identical to that of the hydrochloride salt, as determined by X-ray diffraction crystallographic analysis.[48] In contrast, however, the disulfide dimer of homocysteine, homocystine, is susceptible to oxidation by hydrogen peroxide, forming homolanthionine sulfone, homolanthionine sulfoxide, homocysteic acid, and sulfate.[12]

The unusual chemical reactivity of homocysteine thiolactone is demonstrated by analysis of the products of the reaction of weak alkali with its hydrochloride salt.[18] Under these conditions, oxidation fails to yield homocystine, as expected, but instead a high melting, amorphous polymeric substance with solubility properties similar to those of keratin is obtained. This substance was found to be a polymer formed by oxidation of the sulfhydryl groups of homocysteine diketopiperazine. In contrast, hydrolysis of homocysteine thiolactone hydrochloride with strong alkali results in the quantitative formation of homocysteine, which is oxidized directly to homocystine without the intermediate formation of homocysteine diketopiperazine polymer. These findings are summarized in Figure 1.

Dimerization of homocysteine thiolactone in the presence of weak alkali to form homocysteine diketopiperazine is the result of reaction of one molecule of homocysteine thiolactone with the free amino group of another molecule, forming a peptide bond. The resulting homocyteinyl homocysteine thiolactone forms a second internal peptide bond by opening of the second thiolactone ring to form homocysteine diketopiperazine. This substance contains two free sulfhydryl groups that are oxidized by atmospheric oxygen to form the insoluble homocysteine diketopiperazine disulfide polymer. If homocysteine thiolactone hydrochloride is hydrolyzed in the presence of dichloromethane, the free base form of homocysteine thiolactone is extracted into the dichloromethane layer and isolated in pure form after drying and evaporating the solvent.[52] The resulting colorless, viscous oil is freely soluble in both water and organic solvents. The oil has a pungent, sulfurous, fishy, amine odor. The oil spontaneously forms an insoluble white solid, when allowed to stand for one hour in the presence of air at room temperature. The nuclear magnetic resonance (NMR) spectrum of the oil is similar to that of homocysteine thiolactone salts. Oxidation of this oil in aqueous solution by aeration results in formation of the high melting amorphous diketopiperazine polymer. These reactions and hydrolysis conditions are illustrated in Figure 1.

Understanding of the scope of reactivity of homocysteine thiolactone was extended in 1956, when formation of peptide bonds was shown to occur by ammoniolysis of homocysteine thiolactone by amines and by amino acids.[3] This reaction introduces thiol groups into proteins by homocysteinylation of free amino groups, a process called thiolation.[4] The reaction of immunoglobulin G with homocysteine thiolactone introduces peptide bound homocysteinyl thiol groups, which form mercaptides by reaction with methyl mercury.[34] Homocysteinylated immunoglobulin G labelled with mercury in this way can be visualized in tissues by electron microscopy.[35] The same reaction was later employed with low density lipoprotein (LDL), and the affinity of thiolated LDL for membrane receptors of leukemic lym-

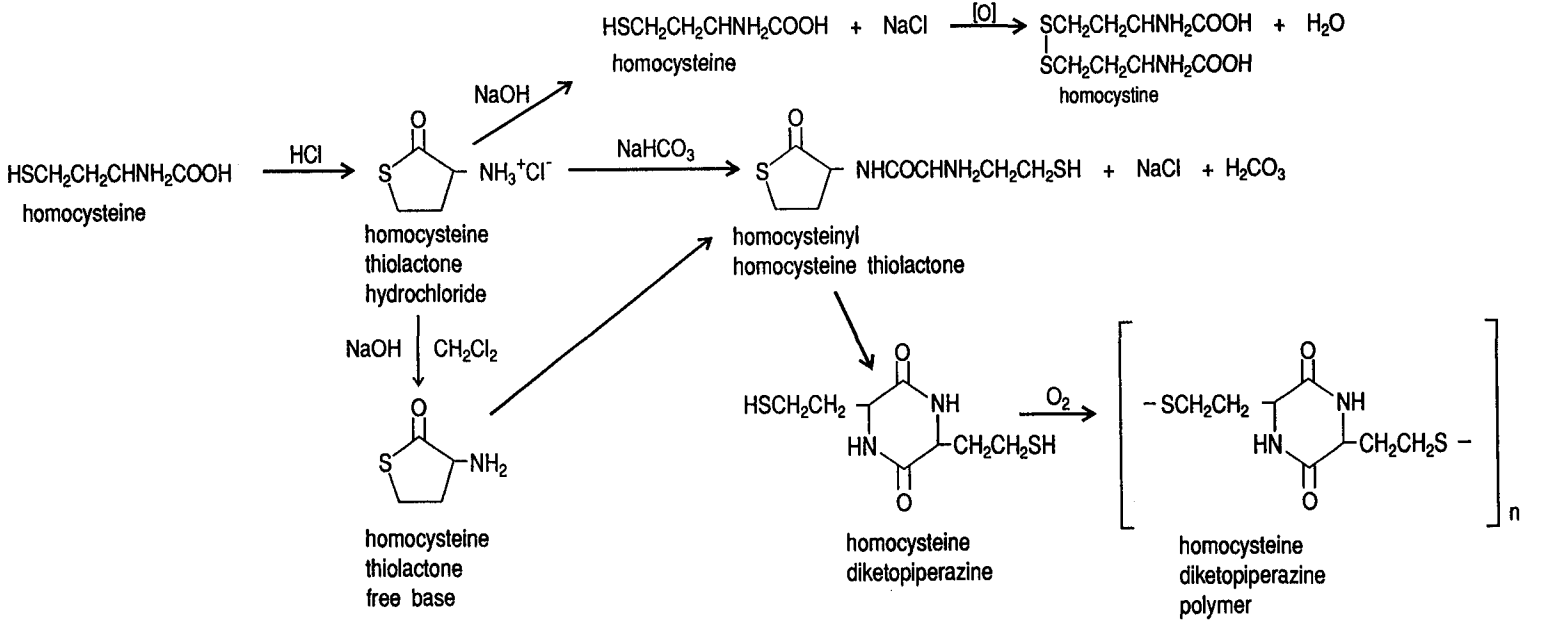

FIGURE 1. Chemical synthesis, hydrolysis, oxidation, and polymerization reactions of homocysteine thiolactone are indicated.

phocytes was found to be preserved.[94] These studies show that homocysteine thiolactone introduces peptide bound homocysteinyl groups by reaction with the free amino groups of diverse proteins.

Biochemical Functions of Methionine

An important derivative of methionine was discovered by Cantoni in 1952, as the product of the enzymatic reaction of adenosine triphosphate (ATP) with methionine to form S-adenosylmethionine.[6] This optically active sulfonium derivative is synthesized in all living cells by adenosyl transferase with the intermediate formation of tripolyphosphate.[55] Adenosyl methionine transfers its methyl group to a wide variety of methyl acceptor compounds, with the formation of adenosyl homocysteine and methylated acceptor. Although homocysteine is formed within cells by enzymatic hydrolysis of adenosyl homocysteine, its concentration is very low because the reaction with adenosine favors formation of adenosyl homocysteine[13] and because homocysteine is actively remethylated to methionine by methyltetrahydrofolate homocysteine methyl transferase. This enzyme requires methyl cobalamin for activity.[96] In addition, adenosyl methionine, which is an allosteric activator of cystationine synthase and inhibitor of methylenetetrahydrofolate reductase, further prevents accumulation of intracellular homocysteine by coordinate regulation of remethylation and transsulfuration of homocysteine.[79]

Adenosyl homocysteine is a potent inhibitor of the many methyl transfer enzymes that require adenosyl methionine. The ability of animals to grow without dietary methionine, using homocysteine and a methyl donor, is explained by the active remethylation of homocysteine to methionine by methyltetrahydrofolate homocysteine methyl transferase or by betaine methyl transferase. Betaine arises by oxidation of choline, and betaine methyl transferase, a liver enzyme, catalyzes remethylation of homocysteine to methionine by transfer of the methyl groups of betaine. Several important reactions in the metabolism of methionine are illustrated in Figure 2.

These nutritional and biochemical studies of sulfur amino acid metabolism demonstrate the vital importance of methionine as an essential amino acid in protein synthesis, in methylation reactions, and in formation of methionine from homocysteine and methyl donors by transmethylation. Furthermore, methionine is an essential structural component of proteins, and methionyl transfer ribonucleic acid (RNA) functions as the initiator of protein synthesis by polyribosomes. Methionine is the only essential amino acid that contains sulfur, and cysteine is nutritionally non-essential, since is is readily formed from methionine by synthesis and catabolism of cystathionine.

Altered methionine metabolism is encountered in malignant cells, in atherosclerosis, and in somatic growth of animals. However, the clue to understanding of the vital importance of homocysteine thiolactone in controlling aspects of these disease processes and normal cellular functions arose from an unusual source, the study of pathological findings associated with inborn errors of sulfur amino acid metabolism. Analysis of metabolic and pathological abnormalities in children with homocystinuria caused by different inherited enzymatic disorders of homocysteine metabolism suggested a series of experiments in organic synthesis, biochemistry physiology, cell biology, and experimental pathology. The results of these studies have helped to elucidate the role of homocysteine thiolactone metabolism in cellular respiration, in differentiation of normal cells and tissues, and in atherosclerosis, cancer and other chronic degenerative diseases.

Homocystinuria and Arteriosclerosis

In 1933, a mysterious case of carotid arteriosclerosis and stroke in childhood was presented at the Clinical Pathological Conference of the Massachusetts General Hospital.[10] An eight-year-old mentally retarded boy with congenital dislocation of the optic lenses and coxa vara developed headache, vomiting, obtundation, and left hemiparesis. The symptoms progressed to coma and death on the third hospital day. Autopsy revealed severe arteriosclerosis of the carotid arteries with acute thrombosis and cerebral infarct. Dr. Tracy Mallory, the pathologist who presented the autopsy findings, commented that the "very marked intimal thickening [of the carotid artery] could be the result . . . [of a] sclerotic process such as one sees in elderly people." The disease process was considered to be of congenital origin, involving optic lenses, brain, and carotid arteries. Not until 1970 was the correct diagnosis proven by demonstration of homocystinuria and cystathionine synthase deficiency in the relatives of the child discussed in 1933.[80]

Homocystinuria was discovered in the early 1960s.[9,21,82] DuVigneaud's group had previously identified several patients with homocystinuria at the New York Hospital in the 1950s, but these cases were not published or investigated more extensively.[11] Deficiency of cystathionine synthase is found in liver biopsies[56] and in cell cultures from the skin of patients with homocystinuria.[92] Pathological study of the blood vessels in fatal cases reveals frequent arterial and venous thrombosis of major branches, as well as fibrous intimal plaques of coronary, carotid, renal and other arteries.[8,23,78] Subsequent clinical study showed that patients responding to pyridoxine therapy survived longer than non-responders and that cardiovascular disease caused over two-thirds of the 64 fatalities observed in 629 patients.[58]

In 1969, a different type of homocystinuria, accompanied by cystathioninuria, hypomethioninemia, and methylmalonic aciduria, was found to be caused by deranged cobalamin metabolism.[39,57] Pathological study of this case revealed widespread arteriosclerotic changes of major arteries, focal infarction and metabolic encephalopathy of frontal and parietal lobes, fatty liver, and atrophic and metaplastic changes of gastric mucosa.[40,47] Since elevated blood homocysteine was the only metabolic abnormality found both in cystathionine synthase deficiency and in methyl transferase deficiency, arteriosclerotic lesions in these inborn errors of metabolism were attributed to the effects of homocysteine derivatives on arterial cells and tissues.[40] This interpretation was subsequently supported by the finding of similar arteriosclerotic lesions in a child with a third type of homocystinuria caused by deficiency of methylenetetrahydrofolate reductase.[33,59] The enzymatic deficiencies associated with the three principle types of homocystinuria are illustrated in Figure 2.

Homocysteine Theory of Arteriosclerosis

The association of arteriosclerotic lesions with homocysteinemia caused by different inherited disorders of homocysteine metabolism is the key observation[40] that led to the development of the homocysteine theory of arteriosclerosis.[42,54] Since discovery of the homocysteine theory, the atherogenic effect of homocysteine accumulation has been established through several lines of investigation. Experimentally induced homocysteinemia in animals causes arteriosclerotic lesions similar to those observed in children with homocystinuria and similar to those observed in individuals without known enzymatic disorders.[42] Study of patients with cerebral, coronary, and peripheral atherosclerotic disease has

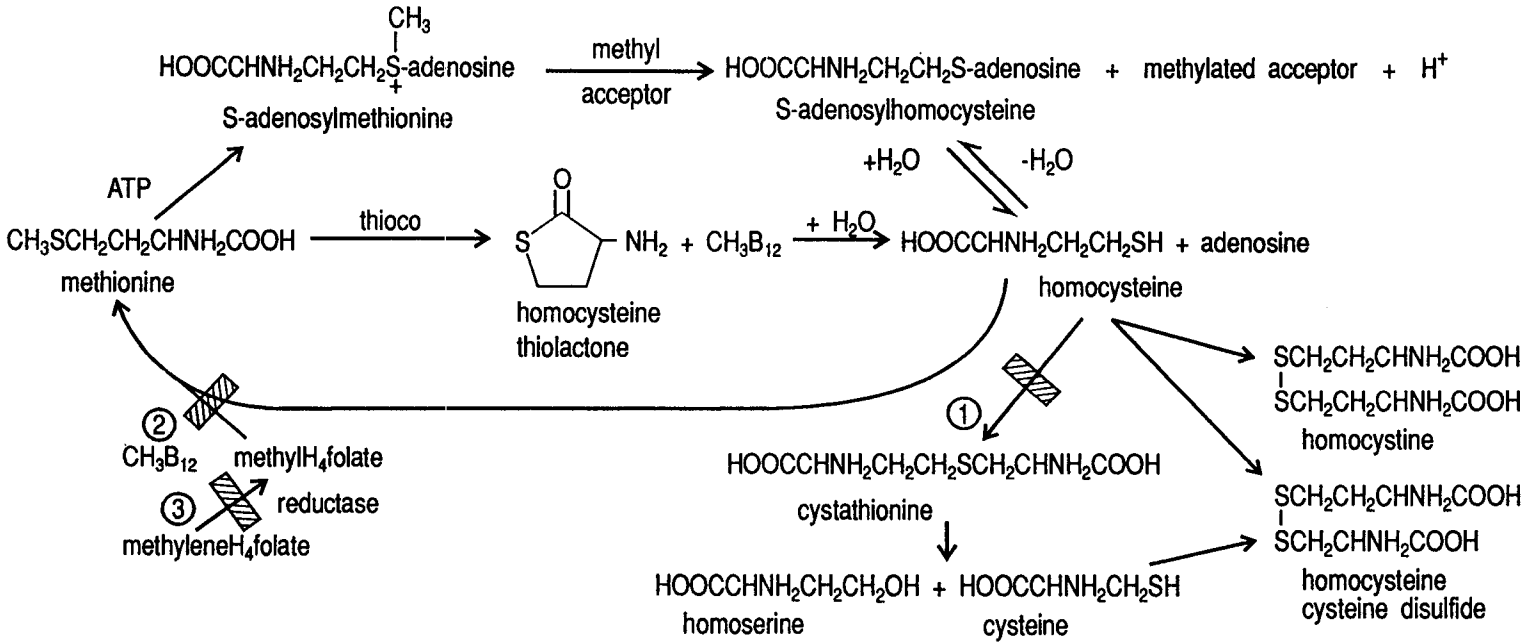

FIGURE 2. Biochemical pathways of synthesis of homocysteine thiolactone, adenosyl methionine and homocysteine are indicated. Inherited deficiencies of ① cystathionine synthase, ② methyltetrahydrofolate homocysteine methyl transferase, and ③ methylenetetrahydrofolate reductase cause accumulation of homocystine and homocysteine cysteine disulfide in tissues and body fluids.

demonstrated elevated blood homocysteine levels, compared to normal controls.[91] Drugs, such as azaribine, methotrexate, nitrous oxide, anti-convulsants, and diuretics, as well as environmental toxins such as tobacco smoke and carbon disulfide, which antagonize pyridoxal phosphate, cause elevated blood homocysteine and increased atherogensis.[46] Hormonal abnormalities, such as thyroid deficiency and oral contraceptives, as well as chronic renal failure, also cause elevated blood homocysteine.[46] A thermolabile, inherited defect in methylenetetrahydrofolate reductase is associated with homocysteinemia and coronary heart disease.[31] Approximately one-fourth of individuals without known risk factors who have carotid, peripheral, or coronary atherosclerosis and homocysteinemia are heterozygous for cystathionine synthase deficiency.[91] A prospective study of 14,916 male physicians showed that hyperhomocysteinemia is associated with increased risk of myocardial infarction.[85] Hyperhomocysteinemia is now generally accepted as an independent risk factor for coronary heart disease[20] and other forms of occlusive vascular disease.[32]

The homocysteine theory of arteriosclerosis explains epidemiological features of atherosclerosis in susceptible and in resistant populations.[42,46] The consumption of diets with predominantly animal protein from meat and dairy products, together with abundant refined nutrients, sugar and fats, and highly processed foods, is associated with a high risk of atherosclerosis in susceptible populations. The consumption of diets with predominantly plant proteins from grains, legumes, vegetables and fruits, together with abundant fresh, unrefined, unprocessed foods without added sugar and fats, is associated with a low risk of atherosclerosis in resistant populations. Proteins of animal origin have a methionine concentration two to three times greater than proteins of plant origin. The quantity of essential micronutrients required for metabolizing methionine and preventing homocysteine accumulation, especially folate, pyridoxine, cobalamin, riboflavin, and choline or betaine, is also correlated with decreased risk of atherosclerosis. Thus, the methionine to micronutrient ratio of the diet is high in susceptible populations and low in resistant populations.[14] Furthermore, the blood homocysteine levels of South African indigenous populations that are resistant to atherosclerosis are considerably lower than the blood homocysteine levels of urban populations that are susceptible to the disease.[87,88]

Analysis of Blood Homocysteine

Analysis of blood homocysteine in clinical or population samples is accomplished by amino acid analysis chromatography, high performance liquid chromatography, gas chromatography, enzymatic analysis, or other methods.[46,91] Free homocysteine is determined as homocysteine cysteine disulfide and homocystine in serum or plasma from which protein has been precipitated and removed by sulfosalicyclic acid or trichloroacetic acid. Homocysteine bound to protein by disulfide bonds is determined by chromatography following reduction with dithiothreitol, sodium borohydride, or tributyl phosphine, and subsequent precipitation of plasma proteins. A recent method for homocysteine analysis by high performance liquid chromatography is designed for screening clinical samples in hospital laboratories.[89] Acid hydrolysis of whole serum results in higher levels of homocysteine in serum from patients with coronary heart disease, compared to disulfide bound homocysteine.[64,68] If a low volume ratio of serum or plasma to acid is employed during hydrolysis, diminished[1] or undetectable[15] levels of homo-

cysteine are observed. The increased recovery of homocysteine by acid hydrolysis with a high volume ratio (1:1) of serum to acid[66,68] is attributed to release of peptide bound homocysteine from thiolated proteins.[53] Peptide bound homocysteine originates from reaction of endogenous homocysteine thiolactone with amino groups of serum protein.[4]

Attempts to isolate free homocysteine thiolactone from lipoproteins by a chloroform-methanol extraction method were unsuccessful, presumably because the quantities are below the limits of detection.[44] However, increased quantities of homocysteine were demonstrated in low density lipoprotein (LDL), very low density lipoprotein (VLDL), and high density lipoprotein (HDL) fractions of hypercholesterolemic men, compared to normal controls, by the acid hydrolysis method.[65] Homocysteine thiolactone hydrochloride added to serum was shown to be recovered as homocysteine cysteine disulfide and homocystine following acid hydrolysis.[66] In hypercholesterolemia, the atherogenic index for homocysteine, LDL_{HCy}/HDL_{HCy}, is 3.5 times greater than in controls, and the atherogenic index for cholesterol, LDL_{Chol}/HDL_{Chol}, is 2.2 times greater than in controls.[65] These findings show that in hypercholesterolemia, appreciable quantities of homocysteine are carried by LDL in the form of homocysteinyl groups bound by peptide bonds to the amino groups of apoB. The source of these homocysteinyl groups is endogenous homocysteine thiolactone, originating from methionine in liver.[83]

Modification of LDL by Homocysteine Thiolactone

Reaction of homocysteine thiolactone with LDL introduces homocysteinyl groups by thiolation of free amino groups of apoB protein.[94] Reaction of freshly synthesized homocysteine thiolactone free base with LDL causes immediate aggregation and precipitation of thiolated LDL.[43] Other lipoprotein fractions are not precipitated by homocysteine thiolactone free base. Thiolation of LDL by homocysteine thiolactone increases internalization of LDL by membrane receptors, degradation and cholesterol accumulation within cultured macrophages.[63] Thiolation of LDL by homocysteine thiolactone is accompanied by an increase in density, increase in electrophoretic mobility, and a decrease in the number of free amino groups of apoB protein.[63] Thus, thiolated LDL becomes aggregated and susceptible to spontaneous precipitation because of interaction between homocysteinylated amino groups, probably by disulfide and by diketopiperazine formation.[63,94]

Chemical or biological modifications of lipoproteins, particularly LDL, are believed to increase their atherogenicity.[86] Modification of LDL by acetylation, acetoacetylation, or carbamylation increases uptake and cholesterol deposition within cultured macrophages.[24] Incubation of LDL with cultured endothelial cells, smooth muscle cells or macrophages results in oxidation of LDL by a peroxidation process involving superoxide, hydroxyl radical, and hydroperoxyl radical, generated by cultured cells in the presence of transition metal ions.[28] During this process, phosphatidyl choline is hydrolyzed to lysophosphatidyl choline, esterified cholesterol is decreased, and fatty acids are oxidatively degraded to malondialdehyde, hydroxynonenal, and other short chain aldehydes.[61] Thiolation of LDL by homocysteine thiolactone does not increase susceptibility to oxidative modification *in vitro*.[63] However, thiolated LDL is internalized by binding to LDL receptors and by phagocytosis within macrophages. Release of homocysteine from internalized, thiolated LDL by hydrolytic degradation within macrophages presumably leads to oxida-

tive degradation of lipids through effects on cellular metabolism.[63] Furthermore, low density lipoprotein of increased density has increased susceptibility to oxidative modification by macrophages,[62] presumably because of increased thiolation of apoB protein by homocysteine thiolactone.[63,65]

Oxidation, Homocysteine and Atherogenesis

Several oxidized derivatives of cholesterol, especially 25-hydroxy cholesterol and cholestane triol, have been shown to be highly atherogenic.[71] Purified cholesterol is readily oxidized by atmospheric oxygen and is non-atherogenic in animals, when protected against oxidation. Hence, the atherogenic effect of cholesterol in experimental atherogenesis is attributable to its cholesterol oxide content.[29] The high incidence of atherosclerosis in certain immigrant populations in England is attributed to the atherogenic effect of cholesterol oxides in the heated, clarified butter in their diets.[30] Other processed and preserved foods containing cholesterol, such as spray-dried egg yolk, bleached butter oils and cheeses, and foods that are deep fried in fat, contain angiotoxic cholesterol oxides. Several of these cholesterol oxides have been demonstrated within atherosclerotic plaques, but the origin of these compounds, whether from dietary sources or from oxidation of lipids within artery wall, is less certain.[71] Many of these cholesterol oxides are quite toxic to cultured cells and carcinogenic in animals, but the molecular basis for this toxicity is incompletely understood.

Homocysteine is toxic to cultured endothelial cells, and the toxicity is associated with hydrogen peroxide formation and facilitated by copper ions.[84,95] Homocysteine is more toxic to cultured cells deficient in cystathionine synthase than normal cells, and the toxicity is reversed by pyridoxine in cells cultured from homocystinuric patients who respond to pyridoxine therapy.[41] These observations show that cellular homocysteine accumulation is associate with toxic effects and that the cystathionine synthase transsulfuration pathway of normal cells reduces toxicity by cystathionine formation from homocysteine. A related observation is that peroxidation of LDL solutions *in vitro*, in the absence of cells, is facilitated by homocysteine, cysteine, mercaptoethanol or reduced glutathione, leading to binding of oxidized LDL to acetyl LDL receptors and internalization by cultured macrophages.[70] Thiol compounds are well known facilitators of lipid peroxidation by oxygen and transition metal ions. In fact, men with high stored iron and increased serum ferritin have an increased risk of myocardial infarction,[76] suggesting increased atherogenesis from peroxidation of lipoproteins and cholesterol by ferric ions and by homocysteine released from homocysteinylated LDL within artery wall.[63]

A considerable body of evidence suggests that progression of arteriosclerosis is associated with and promoted by accelerated oxidation of cellular and tissue constituents by free radical oxidants.[22] Radical scavanging antioxidant nutrients, such as tocopherol, selenium, carotenoids, retinoids, and ascorbate, may diminish progression of arteriosclerosis by counteracting the atherogenic effect of free radical oxidants. Cellular enzymes, including superoxide dismutase, catalase, and glutathione peroxidase, also counteract free radical oxidants by catalysis to less reactive oxygen species. These enzymes convert superoxide to hydrogen peroxide and oxygen, hydrogen peroxide to water and oxygen, and hydrogen peroxide to water and oxidized glutathione, respectively. Both epidemiological and experimental evidence suggest that suppression of free radical oxidation by antioxidant nutrients and antioxidative enzyme activity also suppresses atherogenesis.[22]

For many years the dietary consumption of unsaturated oils from plants and from fish has been known to be associated with decreased risk of atherogenesis, compared to dietary consumption of saturated fatty acids. The molecular basis for this protection is believed to involve prevention of the accumulation of peroxidized lipids and cholesterol oxides in LDL and cell membranes.[22] The n-6 unsaturated fatty acids of plant oils and the n-3 unsaturated fatty acids of fish oils are believed to explain the protective effect of consumption of plant foods and fish against atherogenesis.[37] This view is supported by the antiatherogenic effects of antioxidant nutrients and drugs, such as probucol, in experimental animals.[7,36] Blood homocysteine is decreased by feeding corn oil in a synthetic diet to rabbits, compared to the same diet with butter, resulting in a blood homocysteine level similar to that observed with the control diet.[50] Blood homocysteine is also decreased by fish oil supplements, compared to olive oil supplements, in hyperlipemic men.[66] These studies show that unsaturated fatty acids of plant and fish oils decrease the level of blood homocysteine, explaining their antiatherogenic effects in experimental animals and in populations resistant to arteriosclerosis.

Homocysteine Thiolactone, Cholesterol and Lipoproteins

Administration of homocysteine thiolactone hydrochloride to experimental animals by parenteral routes causes fibrous arteriosclerotic plaques in the aorta and arteries of rabbits[51,54] and baboons.[26] When a synthetic diet with saturated fat and cholesterol is also fed, the fibrous arteriosclerotic plaques induced by parenteral homocysteine thiolactone are converted to fibrolipid plaques.[50] High doses of homocysteine thiolactone also cause elevation of blood cholesterol, LDL and VLDL,[19] but no changes are found in HDL.[50] Parenteral thioretinamide causes elevation of blood homocysteine, cholesterol, and LDL + VLDL, correlating with increased atherogenesis.[50] Thioretinaco causes elevation of blood homocysteine and increased atherogenesis without changes in blood lipids, compared to the effect of the atherogenic diet. Notably, in these experiments high dietary cholesterol and saturated fat from butter are not atherogenic, probably because the synthetic diet was protected against oxidation, preventing formation of angiotoxic cholesterol oxides within the synthetic diet.

Since the early 20th century, many studies have documented increased atherogenic risk with elevated blood cholesterol, with elevated LDL, and with decreased HDL levels.[81] In a recent autopsy study, blood cholesterol was found to be correlated with severity of atherosclerosis, but in two-thirds of cases with severe atherosclerosis, the disease developed without evidence of elevated serum cholesterol, diabetes, or hypertension.[45] A study of the effect of micronutrients on coronary heart disease showed that pyridoxine, folate, cobalamin, riboflavin, choline, and troxerutin decreased not only blood homocysteine but also cholesterol, LDL, and apoB.[69] These studies show that both elevated blood homocysteine and elevated cholesterol and LDL are risk factors for atherogenesis and that specific micronutrients and antioxidants may diminish or prevent the disease by preventing homocysteine accumulation. A study of men with hyperhomocysteinemia revealed deficiencies of serum folate, vitamin B_{12}, and vitamin B_6 in 25 to 59 percent of subjects, and elevated serum homocysteine was reduced to normal by oral supplements of these micronutrients.[90]

Prominent inhibition of atherogenesis is produced by intravenous infusion of free amino acids and other nutrients in rabbits, and total parenteral nutrition therapy causes regression of atherosclerotic plaques in human patients.[16] This

antiatherogenic and regressive effect of intravenous amino acids on atherosclerosis is associated with dramatic lowering of plasma cholesterol levels. Excess free amino acids may reduce thiolation of low density lipoproteins by competition for homocysteinylation by endogenous homocysteine thiolactone. This possibility needs to be studied by demonstration of homocysteinylated free amino acids of plasma and decreased homocysteinylation of LDL in these animals and patients.

An interesting regressive effect of intra-arterial hydrogen peroxide infusion on human atherosclerosis was observed in the course of radiation therapy of malignant disease in patients with pelvic neoplasms.[93] Autopsy of several of these patients revealed "a decrease in the number and severity of atheromatous plaques and an increase in flexibility and elasticity of the vessel" distal to the tip of the intra-aortic catheter, compared to proximal areas of aorta. This observation suggests that hydrogen peroxide infusion may interfere with the atherogenicity of homocysteinylated LDL by facilitating homocysteine catabolism within arterial wall cells.

Homocysteine and Thrombosis

Extremely low concentrations of homocysteine thiolactone free base cause aggregation and release of thromboxane and prostacyclin metabolites from normal human platelets.[49] The hydrochloride of homocysteine thiolactone, homocystine, and homocysteine are inactive, but thioretinamide also causes platelet aggregation. Paradoxically, there is no concomitant release of ATP during aggregation of platelets by homocysteine thiolactone free base or by thioretinamide. Homocysteine reduces protein C activation by arterial and venous endothelial cells,[74] and homocysteine enhances endothelial cell factor V by inducing a protease that activates the procofactor to Va.[75] High concentrations of homocysteine inhibit thrombomodulin surface expression by endothelial cells, as well as inhibiting protein C activation.[38] These studies indicate some of the metabolic changes in platelet function and blood coagulation factors, induced by homocysteine, that may explain the increased risk of thrombosis in patients with homocystinuria and in atherosclerotic patients with elevated blood homocysteine.

Thiolation of LDL by homocysteine thiolactone introduces homocysteinyl sulfhydryl groups that may react with the sulfhydryl group of the kringle domains of apo(a), forming lipoprotein(a) by oxidation to disulfide bonds.[67] This formulation needs to be proven by further investigation. Low concentrations of homocysteine increase binding of lipoprotein(a) to fibrin, linking homocysteine accumulation with thrombosis and atherogenesis.[27] In this way, low concentrations of homocysteine may increase the atherogenicity of low density lipoprotein by forming lipoprotein(a) in persons with a genetic predisposition to high levels of apolipoprotein(a).[77]

Homocysteine and Arteriosclerosis

Although the atherogenic effect of homocysteine accumulation is well established in homocystinuria caused by enzyme deficiencies and in experimental animals, important questions concerning the molecular basis for atherogenesis remain to be answered. The molecular basis for intimal injury, the relation of homocysteine accumulation to oxidation reactions, the role of homocysteine in thrombogenesis, and the metabolic changes induced by homocysteine that cause increased cholesterol and lipid synthesis, all need further explanation by consideration of homocysteine thiolactone metabolism.

As will be explained in Section II of this review, the depletion of thioretinaco from somatic cells, especially from hepa-

tocytes, by etiological factors for atherogenesis is believed to increase homocysteine thiolactone formation from methionine and to result in highly homocysteinylated LDL. Cholesterol and fatty acid synthesis are probably increased because of inhibition of oxidative phosphorylation, accumulation of excess acetyl CoA, and increased lipid synthesis within liver. This increased lipid synthesis, in turn, leads to increased secretion of homocysteinylated LDL into hepatic sinusoids. Circulating homocysteinylated LDL affects intimal cells of arteries in areas of turbulence and high pressure, promoted in some cases by systemic hypertension or by familial hypercholesterolemia. The homocysteinylated LDL aggregates are internalized by phagocytic cells of artery wall through interaction with LDL receptors and by phagocytosis, leading to lipid and cholesterol deposition and release of homocysteine thiolactone within arterial intima. Intimal necrosis probably occurs at the side of homocysteine thiolactone release because of increased conversion of thioretinaco to thioco (Section II, Figure 4), depletion of thioretinaco from intimal cells, and an increased thioco/thioretinaco ratio, thus inhibiting oxidative phosphorylation (Section II, Figure 6) and leading to accumulation of reactive oxygen radical species. The increased thioco/thioretinaco ratio within intimal cells probably stimulates mitosis and clonal growth of adjacent medial smooth muscle cells, altering contact inhibition of growth, releasing growth factors, and promoting migration into and hyperplasia of these cells within intima.

Fibrous arteriosclerotic plaques are formed by increased collagen and sulfated glycosaminoglycan synthesis by hyperplastic intimal smooth muscle cells. Increased sulfation of glycosaminoglycans of arterial intima occurs through increased conversion of homocysteine thiolactone to phosphoadenosine phosphosulfate (Section II, Figure 9), causing decreased solubility and increased aggregation of extracellular matrix. The sulfated extracellular matrix binds the LDL molecules which have increased access to intima and media because of increased permeability, resulting from intimal injury by homocysteinylated LDL. Calcification occurs by deposition of calcium appatite within sulfated extracellular matrix and within excess collagen deposits of developing plaques. Fragmentation of elastica interna occurs because of tetrahydrothiazine formation between lysyl aldehyde groups of tropoelastin and homocysteine, inhibiting formation of desmosine and isodesmosine intramolecular cross links. Cholesterol crystals are formed from free cholesterol, resulting from hydrolysis of cholesterol esters liberated from phagocytosed LDL and from LDL-glycosaminoglycan complexes within developing fibrolipid plaques.

The sites of intimal injury produced by circulating homocysteinylated LDL are associated with adherent platelet aggregates that release growth factors and prostaglandins, promoting cellular hyperplasia and mural thrombosis. The homocysteine thiolactone released from phagocytosed homocysteinylated LDL at the site of injury promotes platelet aggregation by effects on platelet membranes. The thrombogenic effect of homocysteine at the site of intimal injury may be potentiated by protein C activation, increased lipoprotein(a) formation, and effects on other plasma and tissue coagulation factors. The fibrin deposited within and over the surface of developing fibrous and fibrolipid plaques contributes to the protein precipitates found within atheromas. Atheromas also contain protein from LDL deposited in fibrolipid plaques and other homocysteinylated serum proteins with altered antigenic structure.

The increased concentration of reactive oxygen radical species produced at the site of intimal injury by homocysteinylated LDL peroxidizes cholesterol and

unsaturated fatty acids of membrane lipids, forming highly atherogenic cholesterol oxides, such as 25-hydroxy cholesterol, cholestane triol, 7-hydroxy cholesterol, and cholestanone.[71] The accumulation of peroxidized cholesterol and unsaturated lipids at the site of intimal injury alters the membrane structure, forming elevated intimal lesions[72] and leading to further depletion of thioretinaco from affected intimal cells. Peroxidation of membrane lipids leads to increased production of malondialdehyde and deposition of malondialdehyde altered protein within atheromas.[25] Peroxidation of membrane lipids of cellular organelles leads to lysosomal activation and increased release of degradative enzymes that promote lipid and cholesterol deposition within atheromas.

The foregoing description is believed to include the principal pathophysiological processes by which altered homocysteine thiolactone metabolism produces the fibrous plaques, fibrocalcific plaques, fibrolipid plaques, lipid streaks, atheromas, and mural thrombi that characterize atherosclerosis. Formation of atherosclerotic aneurysms occurs when intraluminal blood pressure expands an area of arterial wall weakened by destruction of elastic fibers, loss of elasticity, and diffuse fibrosis. Progressive narrowing of arteries occurs by gradual encroachment of enlarging fibrocalcific plaques and atheromas upon arterial lumens. Sudden occlusion of arteriosclerotic arteries occurs when an occlusive thrombus forms at sites of narrowing or when hemorrhage occurs within fibrolipid plaques or atheromas. Recanalization of thrombi occurs by organization of occlusive thrombi or by angiogenesis within hemorrhagic atheromas and fibrolipid plaques. The diffuse fibrous intimal plaques of arterioles, which are associated with atherosclerotic plaques of aorta and muscular arteries in many cases, probably occur through similar pathophysiological processes.

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